APPLICATION

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TITLE: HYBRIDIZATION PROBE

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DESCRIPTION

HYBRIDIZATION PROBE

Technical Field

The present invention relates to a labeled DNA and a method for detecting genes with the labeled DNA.

Background Art

Labeled DNAs have been utilized in a wide variety of analytical methods based on the specific interaction between DNAs having complementary nucleotide sequences. The following DNA-labeling methods are known:

(1) a method in which a radioisotope-labeled phosphate group is introduced into the 5' end of synthetic DNA with T4 polynucleotide kinase;

(2) a method in which biotin or digoxigenin is directly added to the end of oligo DNA by a chemical method, thereby labeling the DNA; or

(3) a method in which a labeled nucleotide is added to an oligo DNA with terminal transferase or the like after the DNA is synthesized.

However, it is difficult to prepare probes having high specific activity by the method (1) or (2) of these methods. Only one label molecule per DNA molecule can be introduced by the methods, because the label is linked to only an end of the DNA molecule according to the methods. Therefore, high specific activity cannot be expected based on the methods.

On the other hand, multiple nucleotides are connected together in the method of (3), which is also referred to as 3'-tailing label method. A number of labeled nucleotides or nucleotide derivatives (hereafter, the term "nucleotide compounds", which means both nucleotides and nucleotide derivatives, is used in some cases) can be introduced into a DNA and can achieve high specific activity. It is sometimes difficult to introduce multiple nucleotide derivatives by this method because of steric hindrance by the nucleotide derivatives to be introduced. However, the problem of steric

hindrance can be overcome by combining, as a spacer, nucleotides with no steric hindrance such as deoxyadenylic acid (dAMP), deoxycytidylic acid (dCMP), deoxyguanylic acid (dGMP), thymidylic acid (dTMP), or deoxyuridylic acid (dUMP). However, the DNA resulting from the reaction could have a different sequence from that of the original one because of the introduced nucleotide compounds and/or the spacer nucleotides. The sequence derived from the nucleotide compounds added can cause additional hybridization, which can be a problem in some cases. An example of nucleotide sequences causing such hybridization is a sequence where a single type of nucleotide is consecutively repeated, which is represented by a poly(A) tail of cDNA. If the signal/noise ratio (hereafter, referred to as S/N ratio) is decreased because of hybridization due to the sequences of nucleotides newly added for labeling, then the sensitivity is not increased even when the specific activity of the probe is higher.

In order to inhibit hybridization due to the sequence of nucleotides newly added for labeling but not derived from the DNA to be labeled, it is necessary to use, as a carrier in the hybridization, homopolymer or random copolymer of deoxyadenylic acid (dAMP), deoxycytidylic acid (dCMP), deoxyguanylic acid (dGMP), thymidylic acid (dTMP), and/or deoxyuridylic acid (dUMP) and to mask the nucleotide sequence responsible for the high levels of background around the signal. Nonetheless, even by this method, it is hard to completely inhibit such hybridization due to the sequences of nucleotides newly added for labeling.

In the procedure for gene expression profiling using current array technologies, cDNA corresponding to an RNA to be tested is labeled and then hybridized to immobilized cDNA or oligo DNA (reverse Northern blotting; Science (1999), 283, 83-87; Nat Biotechnol (1996), 14, 1675-80). Unlike ordinary Northern blotting, this method requires not a labeled probe but a labeled target nucleotide sequence, because it is difficult to achieve sufficient sensitivity in this type of analytical method based on current labeling techniques. This method is excellent to analyze a large number of genes at once. However, when the alteration in expression level is to be determined based on the variation of signal intensity between different arrays,

such arrays to be compared must be identical. Actually, it is difficult to secure such uniformity among multiple arrays. Therefore, it is necessary to normalize the difference between arrays, if two or more types of RNAs are to be compared with each other with different arrays. Thus, when there are two types of RNAs to be compared, they are typically labeled with different fluorescent compounds and competitively hybridized to a single array to observe the alterations in expression level. Probes achieving sufficient sensitivity makes it possible to employ the same principle as used for the usual Northern hybridization, namely a method in which a labeled probe can be allowed to react to immobilized target nucleotide sequences. If such a method is established, the expression levels of multiple genes can be analyzed with a single array, which is expected to potentate the applicability of array technology increasingly.

It is known that inosinic acid can be used as a substrate in the nucleotide amplification reaction to prevent secondary structure formation of amplification products. Formation of the secondary structure is considered to result in the reduction of resolution, e.g., in gel electrophoresis. Thus, it can be expected that the application of inosinic acid improves the sensitivity. This principle has been applied to PCR for DNA synthesis (Proc. Natl. Acad. Sci. USA., 76, 2232-2235) and a system of RNA synthesis (Unexamined Published Japanese Patent Application (JP-A) No. Hei 6-165699. It has also been reported that the application of 7-deaza-2'-deoxyguanosine-5'-triphosphate (c7dGTP) prevents the formation of complicated secondary structure in PCR (WO90/03443). However, the applications shown in these reports are achieved merely for the purpose of inhibiting secondary structure formation in nucleotide amplification products, but are not construed as suggesting the prevention of nonspecific base pairing in hybridization assays. In the structural aspect, these methods are different from the method of the present invention in that some portions were converted to inosine in the nucleotide sequence of target nucleic acids in the previous methods.

Disclosure of the Invention

An objective of the present invention is to provide a labeled DNA that meets the two criteria of both high specific activity and high specificity.

The present inventors have noticed that one of causes for the reduction of hybridization specificity of a DNA labeled by 3'-tailing is originating from sequences of nucleotides or nucleotide compounds added for labeling. As long as the nucleotide sequence consists of a, c, t, g, and u, the nucleotide sequence added for labeling can result in occurrence of hybridization with a nucleotide sequence complementary to the nucleotide sequence. Then, the inventors considered the possibility that the specificity is improved by using nucleotides and nucleotide compounds exhibiting only weak affinity in base pairing as nucleotides and nucleotide compounds to be added for labeling. Further, the present inventors selected nucleotides and nucleotide compounds as substrates for terminal transferase to establish a 3'-tailing label method capable of providing probes having high specific activity, and thereby completed the present invention. Specifically, the present invention relates to the labeled DNA, the method for providing the labeled DNA, and the use thereof as follows:

(1) a hybridization probe in which a nucleotide sequence comprising labeled nucleotides or nucleotide derivatives is added to a DNA to be labeled, the added nucleotide sequence

a) comprising nucleotides and/or nucleotide derivatives having weaker affinity of hydrogen bonding in base pairing with bases of the target nucleotide sequence when compared with those of hydrogen bonding in an a/t pair, in an a/u pair, and in a g/c pair; and

b) being introduced into the DNA to be labeled through nucleotide-adding reaction with terminal transferase;

(2) the hybridization probe of (1), wherein the nucleotides of a) are inosinic acids;

(3) the hybridization probe of (2), wherein the added nucleotide sequence comprises labeled nucleotides or nucleotide derivatives and unlabeled inosinic acids or derivatives thereof;

(4) the hybridization probe of (3), wherein the labeled nucleotides or nucleotide derivatives are labeled inosinic acids or

inosinic acid derivatives;

(5) the hybridization probe of (1), wherein the added nucleotide sequence *per se* is incapable of hybridizing to any nucleotide sequences under stringent hybridization conditions for the DNA to be labeled;

(6) a method for detecting, with the hybridization probe of any one of (1) to (5), a nucleic acid having a nucleotide sequence complementary to the DNA to be labeled;

(7) the method of (6), wherein RNA or cDNA library is detected;

(8) a method for labeling a DNA by 3'-tailing with terminal transferase, wherein nucleotides and/or nucleotide derivatives having weaker affinity of hydrogen bonding in base pairing when compared with those of hydrogen bonding in an a/t pair, in an a/u pair, and in a g/c pair and which can be substrates in nucleotide-adding reaction with terminal transferase are used as substrates;

(9) the method of (8), wherein the nucleotide is deoxyinosine 5'-triphosphate;

(10) the method of (8), wherein the nucleotides and/or nucleotide derivatives having weaker affinity in base pairing are mixed with labeled nucleotides or nucleotide derivatives and used as the substrates;

(11) a kit for synthesizing a hybridization probe, the kit comprising

i) nucleotides and/or nucleotide derivatives

(a) having weaker affinity of hydrogen bonding in base pairing with bases of the target nucleotide sequence when compared with those of hydrogen bonding in an a/t pair, in an a/u pair, and in a g/c pair; and

(b) being introduced into a DNA to be labeled through nucleotide-adding reaction with terminal transferase;

ii) labeled nucleotides or nucleotide derivatives; and

iii) terminal transferase; and

(12) a method for preventing hybridization of a hybridization probe in which a nucleotide sequence comprising labeled nucleotides is added to a DNA to be labeled, the hybridization non-specific to

the sequence of the DNA to be labeled, wherein the nucleotides and/or nucleotide derivatives having weaker affinity of hydrogen bonding in base pairing when compared with those of hydrogen bonding in an a/t pair, in an a/u pair, and in a g/c pair are inserted into the added nucleotide sequence.

The "DNA to be labeled" used herein means a DNA capable of specifically binding with a target nucleotide sequence through hybridization. In general, such a DNA is designated as "probe". The DNA typically comprises a nucleotide sequence complementary to the target nucleotide sequence. Alternatively, the DNA can be designed to be able to hybridize with the target when having some mutated nucleotides. Such a DNA can hybridize to the target nucleotide sequence under stringent conditions, and is long enough to stably maintain the formed duplex without being dissociated even when subjected to washing treatment under typical conditions. This DNA to be labeled can also be used without any special modification as has been used previously. Specifically, for each purpose of detection, the DNA may be a chemically synthesized oligonucleotide or DNA fragment resulting from digestion of plasmid or chromosome. Alternatively, the DNA can be PCR products or cDNA prepared by enzymatic nucleotide synthesis, or a fragment thereof. On the other hand, the target nucleotide sequence can be a DNA, RNA, DNA-RNA hybrid, or the like, and there is no special restriction on the type of nucleotide sequence as long as base pairing can occur with the target nucleotide sequence. The specificity and affinity in the hybridization between the target nucleotide sequence and the probe depend on the type of base responsible for base pairing and on the reaction conditions. Determinants having an impact on hybridization and typical hybridization conditions are summarized below (Molecular Cloning, Cold spring harbor laboratory press, 1989).

Temperature of the reaction solution	68°C
Salt concentration	6x SSC
(20x SSC: 3 M NaCl, 0.3 M sodium citrate)	
pH	7.0
Sodium dodecyl sulfate	0.5%

5x Denhardt's reagent
(composition of 50x Denhardt's reagent:
0.01 g/mL Ficoll type 400, Pharmacia;
0.01 g/mL polyvinyl pyrrolidone;
0.01 g/mL bovine serum albumin Factor V, Shigma)

In the present invention, the hybridization probe is provided by adding a nucleotide sequence comprising labeled nucleotides or nucleotide derivatives to the DNA to be labeled. The nucleotide sequence to be added comprises labeled nucleotides or nucleotide derivatives and has the following features, (a) and (b):

(a) comprising nucleotides and/or nucleotide derivatives having weaker affinity of hydrogen bonding in base pairing with bases of the target nucleotide sequence when compared with those of hydrogen bonding in an a/t pair, in an a/u pair, and in a g/c pair; and

(b) being able to be introduced into the DNA to be labeled through nucleotide-adding reaction with terminal transferase.

The term "nucleotides having weaker affinity of hydrogen bonding in base pairing when compared with those of hydrogen bonding in an a/t pair, in an a/u pair, and a g/c pair" means nucleotides having weaker affinity for all the bases of a, t, g, c, and u than affinity of the respective general complementary bases, t, a, c, g, and a. Alternatively, it can be defined by the expression that the nucleotides have only weaker affinity for any of the bases contained in the target nucleotide sequence than the affinity of the typical partners in complementary base pairing. Further, the term "nucleotide derivatives" means compounds obtained by introducing a functional group into a nucleotide. Further, chemically synthesized nucleotides mimicking the structure of a naturally occurring nucleotide, and nucleotides provided by chemically modifying a naturally occurring nucleotide to reduce the affinity thereof in base pairing are also included in the nucleotide derivatives exhibiting weak affinity in base pairing in accordance with the present invention, when meeting the above-mentioned requirements, (a) and (b).

The method of the present invention is characterized by the nucleotides, nucleotide derivatives, and nucleotide compounds that

can be introduced into a DNA to be labeled through nucleotide-adding reaction with terminal transferase. This is one of requirements for the nucleotide compounds to be utilized in the 3'-tailing labeling method. Such nucleotides that meet the above-mentioned requirement include inosinic acid and xanthylic acid. Among them, inosinic acid is a readily available and a preferred nucleotide in the functional aspect, achieving the effect of the present invention. For example, a nucleotide sequence containing deoxyinosinic acid can be added to the 3' end of a DNA by incubating it with terminal transferase in the presence of deoxyinosine 5'-triphosphate as a substrate.

It is generally believed that inosinic acid can contribute to base pairing with some affinity irrespective of the type of partner base. Because of this, it has been thought that the introduction of inosinic acid to label a sequence, as in the present invention, does not reduce but enhance nonspecific reaction. Actually, however, it is possible to greatly reduce the nonspecific reaction by introducing inosinic acid in a sequence to be added.

The hybridization probe based on the present invention or the method for providing the probe, which essentially contains nucleotides exhibiting weak base pairing, also contains other nucleotides (e.g., a, t, c, g, and u, or derivatives thereof). These nucleotides may be labeled. However, the hybridization should be carried out under conditions where the portion added for labeling cannot hybridize to any sequences when the probe contains nucleotide components other than the above-mentioned nucleotides and/or nucleotide derivatives exhibiting weak base pairing. Preferred conditions are conditions where the nucleotide sequence to be added *per se* cannot hybridize to any nucleotide sequences under stringent hybridization conditions for the DNA to be labeled.

In order to achieve such conditions, it is necessary to increase the proportion of the above-mentioned nucleotides (or nucleotide derivatives) exhibiting weak base pairing in the sequence to be added for labeling. Since the minimum proportion depends on the type of nucleotide, the composition of co-existing nucleotides, and the total length, it is difficult to show the typical range. However, those skilled in the art can empirically determine the proportion based

on the disclosure of the present invention. Any of the nucleotides or nucleotide derivatives added to the DNA to be labeled can be subjected to the labeling. Accordingly, labeled inosinic acid can be used in combination with non-labeled nucleotides. In this case, the spacer can consist of non-labeled nucleotides alone, or alternatively the spacer can also contain non-labeled inosinic acid together with the labeled one. For example, when the labeling by the tailing is carried out with deoxyinosine 5'-triphosphate (a nucleotide with weak affinity) and digoxigenin-labeled deoxyuracil 5'-triphosphate, the latter is used in a proportion of 10% or lower and thereby the conditions of the present invention are successfully achieved.

The terminal transferase to be used in the 3'-tailing labeling method adds nucleotides at random to the 3'end of a DNA without distinguishing the type of base. Therefore, the proportion of bases in the nucleotide sequence to be added should be adjusted based on the types of nucleotides used as substrates and the concentration ratio thereof. A specific example in respect to the proportion is exemplified as follows: when deoxyinosine 5'-triphosphate is used in combination with digoxigenin-labeled deoxyuracil 5'-triphosphate label, the concentration of the former is adjusted to 2 to 10 times excess over that of the latter to expect the improvement of specific activity of the label and the inhibitory effect on the nonspecific reaction. Since labeled nucleotides and derivatives thereof generally tend to be less efficient as substrates in the addition reaction with terminal transferase, an adequate concentration ratio should be determined empirically based on the type of label and the types of nucleotides used in combination. It is impossible to strictly control the nucleotide sequence based on the concentration ratio alone. However, based on the disclosure of the present invention, those skilled in the art can readily determine conditions under which accidentally hybridizable nucleotide repeats is not formed.

On the other hand, it is preferable to use, for example, a label sequence consisting of deoxyinosinic acid alone when it is necessary to increase the composition ratio of nucleotide exhibiting weak base

pairing. Such an embodiment can be achieved by combining labeled deoxyinosinic acid as a labeled nucleotide. When the type of base used is deoxyinosinic acid alone, labeling by 3'-tailing can be performed under conditions where a labeled probe with the highest specific activity can be provided at the optimal concentration ratio between labeled deoxyinosine 5'-triphosphate and non-labeled deoxyinosine 5'-triphosphate.

The 3'-tailing labeling method is already known (Molecular Cloning, Cold spring harbor laboratory press, 1989). Specifically, terminal transferase is allowed to act on DNA (or oligonucleotide) to be labeled in the presence of labeled nucleotide and spacer (non-labeled nucleotide) as substrates. In the present invention, a nucleotide such as deoxyinosine 5'-triphosphate may be added as the spacer nucleotide for the reaction. While the terminal transferase commonly used is derived from calf thymus, there is no particular limitation on the origin. The reaction solution can contain a buffer agent for the optimal pH of the reaction, an agent for maintaining enzymatic activity such as bovine serum albumin, or salts such as cobalt chloride, which provide metal ions required for the expression of the enzyme activity. Terminal transferase (1 to 10 units) is added to the solution and the mixture is incubated at 37°C for about 15 minutes to achieve the addition of nucleotide sequence for labeling.

It is preferable to unite into single reaction conditions to minimize variations of specific activity between the batches. For this purpose, stopping agents for the reaction can be used to control reaction time. For example, when glycogen and ethylenediaminetetraacetic acid (EDTA) are added to the reaction, the enzyme activity of terminal transferase is rapidly dropped and thereby the reaction is terminated.

In the present invention, labeled nucleotides or nucleotide derivatives, as well as nucleotides (or nucleotide derivatives) in the nucleotide sequence to be used for labeling may be any types of nucleotides. Even if the nucleotides for labeling scattered in the nucleotide sequence for labeling are nucleotides capable of forming usual base pairs, they are hardly hybridizable because of the random

arrangement thereof. Accordingly, general nucleotides such as a, t, c, g, or u can be used in combination, or alternatively the labeled nucleotide to be used may also be a nucleotide exhibiting weak affinity in base pairing such as inosinic acid as described above. Labeled nucleotides in a nucleotide sequence used for labeling are exemplified by a nucleotide substituted with ^{32}P. Nucleotide derivatives used for labeling is exemplified by nucleotides with a functional group such as a fluorescent compound or ligand having binding capacity. Compounds derivatizing a nucleotide are exemplified by the following compounds:

Fluorescent compound: fluorescein, rhodamine, aminomethyl coumarin; and

Ligand having binding capacity: digoxigenin, biotin.

A kit for preparing hybridization probes can be provided by pre-arranging items necessary for implementing the 3'-tailing labeling method based on the present invention as described herein. The kit of the present invention essentially contains nucleotides and/or nucleotide derivatives, nucleotide compounds, and terminal transferase and, further, can combine buffer preferably used in the reaction, stopping agent for the reaction, reagents to be used for recovering DNA after the reaction, and such.

The hybridization probe of the present invention can be utilized to detect a variety of nucleic acids. Specifically, the probe can be used widely, e.g., for cDNA screening, detection of genes of pathogenic microorganisms and viruses, or analysis for mutation in oncogene. The hybridization probe of the present invention can be used in various assay formats. Specifically, the probe can be used in any of publicly known assay formats, e.g., in dot-blot hybridization assay to observe the reaction of the probe to a target DNA immobilized on a filter, or in *in situ* hybridization assay to observe the localization of nucleic acids on a fixed tissue section. Some assay methods, in which hybridization probes provided by the 3'-tailing labeling method, are already known (Molecular Cloning, Cold spring harbor laboratory press, 1989). However, the occurrence of hybridization due to the nucleotide sequences newly added for labeling is markedly reduced, and therefore masking treatment with

polynucleotide is unnecessary. In addition, the object of hybridization is not limited to DNA, and RNA can also be the object. Further, the probe can be used to screen colonies and plaques.

Gene expression profiling utilizing array technologies is described below as an application example of the inventive hybridization probe based on the high sensitivity and specificity thereof. With the inventive hybridization probe with high sensitivity and high specificity, it is possible to provide accurate expression profiling for a number of RNAs without normalizing arrays. Further, the inventive hybridization probe can be used to detect single nucleotide polymorphisms (SNPs).

Specifically, RNAs, or the cDNAs thereof to be tested for the expression remain unlabeled and they are spotted as mixtures onto a single support to provide an array. The samples to be used can be cDNAs derived from a variety of tissues, or cDNAs from cells collected with time after some drugs are given to the cells. The array is subjected to hybridization treatment with an excess amount of a probe (first probe) of the present invention, which is specific to the gene to be tested. The signal derived from the probe is detected to analyze the level of expression. Subsequently, the resulting double-stranded nucleotide is exposed to denaturation conditions selected based on the Tm value of the probe and thereby the probe is released from the target nucleotide sequence immobilized on the array. The denaturation conditions may be achieved by controlling temperature. There is a known technology to control hybridization electrically (Nat Biotechnol (1999), 17, 365-370). Subsequently, another probe (second probe) is selected for the next gene. Then, the expression level is analyzed in the same manner. This series of treatments is repeated as desired, depending on the number of genes to be tested. A single array can be used repeatedly in this method, and thus expression profiles for many genes can be analyzed with a high degree of precision. The series of treatments can readily be automated. Thus, it can be found that the use of the inventive hybridization probe can rapidly make gene expression profiling efficient. As the matter of course, it is needless to say that the use of the inventive hybridization probe brings similarly high

efficiency to the analyses of SNP.

The principle of the method as described herein is the same as that of the so-called dot-blot assay with cDNA or RNA. However, the method of the present invention can be characterized mainly by using oligo DNA probes with high sensitivity and high specificity and by performing sequential hybridization of multiple distinct probes to a single array. Instead of array on which cDNA or RNA is immobilized, tissue section can be used as a sample (Nature Med. (1998), 4, 844-847) for the implementation of analyses making the most of these advantageous features.

Brief Description of the Drawings

Figure 1 illustrates a photograph showing the results of hybridization assay using an oligonucleotide labeled by 3'-tailing with deoxyinosinic acid as a spacer in accordance with the present invention. In this figure, dIMP indicates deoxyinosinic acid and dAMP indicates deoxyadenylic acid.

Figure 2 illustrates a photograph showing the results of Northern hybridization using an oligonucleotide probe labeled by 3'-tailing with deoxyinosinic acid as a spacer in accordance with the present invention. In this figure, the single band indicates that the probe specifically detected the target mRNA.

Best Mode for Carrying out the Invention

The present invention is described in more detail below with reference to Examples.

Example 1 Labeling of oligonucleotide

(1) An oligonucleotide (ccctacaaagaaaatggagagcct; SEQ ID NO: 1) specific to a gene (accession number: X75861; deposited in the GenBank sequence database administered by National center for biotechnology information (National institutes of health, USA)) was prepared by chemical synthesis (custom-synthesized by GIBCO). The oligonucleotide (100 pmol) was tail-labeled at the 3' end with terminal transferase. The labeling was performed in a solution (0.2 M potassium cacodylate, 25 mM Tris(hydroxy) aminomethane, 0.25 mg/ml bovine serum albumin, 5 mM cobalt chloride, pH6.6) containing 0.5

mM deoxyinosine 5'-triphosphate (nucleotide with weak affinity) and 0.05 mM digoxigenin-labeled deoxyuracil 5'-triphosphate (labeled nucleotide) or in a control reaction solution containing 0.5 mM deoxyadenine 5'-triphosphate and 0.05 mM labeled deoxyuracil 5'-triphosphate. Terminal transferase (2.5 units/μL) was added to the solutions and the reaction mixtures were incubated at 37°C for 15 minutes.

(2) The reaction solutions were then transferred on ice, and stopping solution was added thereto (final concentrations: 10 μg/ml glycogen, 0.2 mM ethylenediaminetetraacetic acid). Then, lithium chloride at a final concentration of 0.4 mM and 3 volumes of ethanol were added to the solutions, and the mixtures were incubated at -30°C for 2 hours.

(3) The mixtures were centrifuged at 12,000 g to precipitate DNA. The precipitated DNA was washed with 70% ethanol, and then dried. The dried labeled DNA was dissolved in 100 μL of water.

Example 2 Immobilization of target gene

Immobilization of plasmid DNA on nylon membrane was carried out as follows. A target DNA of about 0.1 ng/μL was heated at 96°C for 10 minutes. This DNA sample was then rapidly cooled down with ice. The DNA was spotted in a quantity of 1 μL/dot onto a nylon membrane for nucleotide blotting (Boehringer). The nylon membrane was wetted with 2x SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7) and then irradiated with ultraviolet light by a UV Crosslinker (Stratagene), according to the attached protocol to fix the target gene on the nylon membrane. Target DNAs used included the above-mentioned DNA with the accession number X75861 and other 8 cDNAs containing nucleotide sequences unrelated to this DNA. These cDNAs all had a poly(A) tail at the 3' ends thereof.

Example 3 Hybridization with labeled oligonucleotide

The hybridization was performed as follows. First, nylon membranes were subjected to pre-hybridization. Each nylon membrane was incubated in a hybridization solution (6x SSC, 1%(w/v) blocking solution (Boehringer), 0.1%(w/v) N-lauroyl sarcosine, 0.02%(w/v)

sodium dodecyl sulfate) with 0.1 mg/ml poly(A) or without poly(A) at 68°C for 3 hours.

Subsequently, the nylon membranes were subjected to hybridization treatment with a labeled oligonucleotide. A labeled oligo DNA (5 pmol/ml) was added to the hybridization solution and incubated at 60°C for 12 hours. The nylon membranes were soaked in a washing solution (6x SSC, 0.1% sodium dodecyl sulfate) at 60°C for 15 minutes. The washing treatment was repeated 4 times.

Hybridization of the labeled oligonucleotide was detected by a luminescent method using a lumino-detection kit (Boehringer) according to the attached instruction manual. Briefly, alkaline phosphatase-conjugated anti-labeled digoxigenin antibody was allowed to react to labeled oligo DNA on a nylon membrane, and then luminescent substrate for alkaline phosphatase was added onto the membrane for the luminescent signal. The lumino-detection was carried out by visualizing the signal on an X-ray film.

The results are shown in Figure 1. The oligonucleotide which is labeled by 3'-tailing with deoxyinosine 5'-triphosphate as a spacer is capable of specifically hybridizing to a plasmid containing an insert of the same nucleotide sequence of DNA but not to cDNAs of unrelated nucleotide sequence of DNA. On the other hand, the oligonucleotide which is labeled by 3'-tailing with deoxyadenine 5'-triphosphate as a spacer is also hybridized to a plasmid having an insert of cDNA containing unrelated nucleotide sequence of DNA in a non-specific manner which is not due to the sequence of the DNA to be labeled. It is impossible to inhibit the nonspecific hybridization even by previously masking the plasmid with poly(A) oligonucleotide.

Example 4 Northern hybridization carried out by using as a probe an oligo DNA labeled by tailing with inosinic acid

RNA was prepared as follows. Cells of animal culture cell line, NT2 (purchased from Stratagene; cell culture was performed according to the attached instruction manual), were suspended in RNA extraction buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.6), 0.5% Nonidet P-40, 10 mM vanadyl-ribonucleoside complexes) and allowed to stand

still on ice for 10 minutes. Then, the solution was centrifuged at 10,000 g at 4°C for 15 minutes. The supernatant was removed. Further, an equal volume of proteinase digestion buffer (0.2 M Tris-HCl (pH 7.8), 25 mM EDTA (pH 8.0), 0.3 M NaCl, 2% SDS) was added to give a cell suspension. Proteinase K was added to the suspension at a final concentration of 400 µl/ml and the suspension was incubated at 37°C for 90 minutes. A phenol/chloroform solution was added to the suspension and the aqueous layer was extracted. Further, the phenol/chloroform solution was again added to the aqueous solution. The resulting aqueous layer was extracted and 2.5 volumes of ethanol was added thereto. The solution was centrifuged at 5,000 g at 4°C for 10 minutes. The resulting precipitate was washed with 70% ethanol and then air-dried to obtain a preparation of total RNA. The total RNA was dissolved in H_2O, and incubated at 65°C for 5 minutes. An equal volume of 2x column-loading buffer (1x column-loading buffer: 20 mM Tris-HCl (pH7.6), 0.5 M NaCl, 1 mM EDTA (pH 8.0), 0.1% sodium lauroyl sarcosinate) was added to the solution. The resulting solution was loaded onto a column of oligo-dT cellulose (Collaborative Biomedical Products) pre-swollen with the column-loading buffer, and then the RNA was eluted from the column. The eluted solution was again loaded onto the column. The same treatment was repeated 3 times in total. The column was washed with 5 volumes of 1x column-loading buffer, and 2 volumes of column elution buffer (10 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), 0.05% SDS) were added to the column. The eluted solution was recovered, and 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol were added thereto. The resulting solution was centrifuged at 12,000 g at 4°C for 10 minutes. The precipitate formed was washed with 70% ethanol and air-dried to give mRNA. The obtained mRNA was electrophoresed as follows. The mRNA was dissolved in an electrophoresis sample buffer (4 µl of formamide, 2 µl of formaldehyde, 1 µl of 10x MOPS, 1 µl of H_2O (10x MOPS: 14.9 g/L of MOPS, 6.8 g/L of sodium acetate, 3.7 g/L of EDTA, pH7.0)). The sample was heated at 65°C for 10 minutes and then immediately cooled down on ice. The RNA sample was subjected to electrophoresis with agarose (1 g of agarose, 10x MOPS, 73.3 ml of H_2O, 16.7 ml of formaldehyde). The RNA was transferred from the agarose gel to a nylon filter

(Boehringer) according to a commonly used method (Molecular Cloning. A laboratory manual/2nd edition (1989), p7.46-7.50, Cold Spring Harbor Laboratory Press). Then, the nylon membrane was subjected to ultraviolet light irradiation to fix the RNA on the filter.

An oligodeoxynucleotide (gtcacagaattttgagaccca; SEQ ID NO: 2) specific to EF1α was obtained from GIBCO. Labeling of the oligonucleotide by terminal transferase was performed in the same manner as described above. Hybridization treatment of the EF1α RNA-immobilized filter with the labeled oligodeoxynucleotide and detection of hybridization by chemical luminescence method was performed in the same manner as described above. The results are shown in Figure 2. A single band was detected, which corresponds to the expected size (1.0 kb). As clearly seen in Figure 2, the hybridization probe according to the present invention has sufficient sensitivity and specificity to detect the target mRNA on the blot. Thus, it is confirmed that detection for gene expression profiling and SNP can be achieved based on this principle.

Industrial Applicability

Hybridization due to a nucleotide sequence newly added for labeling can be effectively prevented according to the present invention. Further, the nucleotides used in the present invention can be substrates for terminal transferase, and therefore label can be readily introduced into a nucleic acid sequence by adding the nucleotides as substrates in the 3'-tailing labeling method.

The nucleotides (or nucleotide derivatives) exhibiting weak affinity in base pairing, which are used in the present invention, can function as nucleotides for labeling or as spacer nucleotides, and thus it is possible to achieve the efficient introduction of labeled nucleotides and to provide labeled oligonucleotide with high specific activity. Thus, according to the present invention, both the two objectives are achievable; namely inhibition of nonspecific hybridization reaction and preparation of labeled oligonucleotide with high specific activity. In other words, the S/N ratio in hybridization can be readily improved according to the present invention.